

Monitoring brown trout (*Salmo trutta*) eradication in a wildlife sanctuary using environmental DNA

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Abstract

Restoration of habitats often necessitates the eradication of exotic animals from a specified area. One of the many challenges associated with the removal of introduced animals is determining the distribution and continued presence of individuals in order to efficiently target control operations and minimise any adverse effects associated with removal. We examined the feasibility of using environmental DNA (eDNA) from water samples, relative to more traditional electric fishing, netting and spotlight surveys (i.e., visual observations of the small streams at night), to determine the presence of brown trout. Samples were taken from within the Zealandia Sanctuary near Wellington, New Zealand, before and after treatment with the piscicide rotenone. Using filtration of water samples, we successfully extracted brown trout DNA from water both before and after rotenone treatment. In most cases, DNA presence corresponded to results obtained through netting and spotlight surveys, and in one instance detected the continued presence of trout in a treated stream (which was subsequently confirmed). We conclude that the use of environmental DNA to detect the presence of exotic fish can be a useful tool to assist in the assessment and restoration of aquatic habitats.

Keywords: Brown trout, eDNA, eradication, New Zealand, restoration, *Salmo trutta*

Introduction

Determining the distribution and presence of exotic taxa within a defined area and effective evaluation of eradication efforts are significant challenges for restoration ecology. Environmental DNA (eDNA) is one method that has shown promise for detecting and confirming the presence of introduced species (e.g., Jerde et al. 2011). In such cases DNA is obtained indirectly from environmental samples such as soil or sediment rather than directly from the target species (Ficetola et al., 2008, Jerde et al., 2011, Dejean et al., 2012, Mahon et al., 2013). To date, eDNA has been used to detect species in a diverse range of environments and sample types such as soils (Hofreiter et al., 2003), ice cores (Willerslev et al., 2007) and faeces (Banks et al., 2009). For freshwater vertebrates, DNA from material such as sloughed skin cells or gut epithelium is likely to be present in the environment and has been used to infer the range of introduced silver carp (*Hypophthalmichthys molitrix*), bighead carp (*H. nobilis*) (Jerde et al., 2011), frogs and salamanders (Goldberg et al., 2011) and six species of fish in the Yura River, Japan (Minamoto et al., 2012). However, a more widespread application of the technique awaits further assessment.

In New Zealand, numerous species have been introduced, intentionally or unintentionally, via human activities, many of which have caused marked ecosystem changes (Vitousek et al., 1997, Clout, 1999). Consequently, conservation efforts are often focussed on removing introduced species from selected habitats with the intention of restoring the site to a predefined historical condition (Clout, 1999). One example of an on-going restoration project is the Zealandia Sanctuary (41.290817°S,

174.753377°E), near Wellington. This 252 ha reserve of regenerating shrub land and hardwood forest is intended to reconstruct the local pre-human flora and fauna. Since 1992, a major part of the restoration project has been the removal of introduced animals such as rats and mice from inside the reserve and the building of a pest-proof fence to prevent recolonisation of the sanctuary by introduced mammals (Lynch, 1995). A more recent aspect of Zealandia's restoration is the removal of introduced fish such as brown trout (*Salmo trutta*) from the sanctuary's waterways. The presence of brown trout has been linked with the decline of many New Zealand native fish species either directly by predation or indirectly through competition for food (Townsend, 1996, McDowall, 2003, Townsend and Crowl, 1991, McIntosh et al., 2010). Measures to eradicate brown trout are obviously dependent on detecting the presence of fish. However, brown trout can be difficult to detect visually as they tend to remain motionless and close to cover in rivers (Hicks and Watson, 1985).

The use of eDNA has yet to be widely implemented for conservation management practices in part because there is no standardised technique that is suitable for every ecosystem. Accordingly, the technique has to be individualised for each ecosystem and species of interest (Darling and Mahon, 2011). Confounding factors include the turbidity of the water and the co-extraction of PCR inhibitory chemicals along with the desired DNA from the samples (Bott et al., 2010, Darling and Blum, 2007). Furthermore, little is known about rates of false positives and false negatives from issues such as contamination, misidentification and minimum detectable amounts (Darling and Mahon, 2011). To assess the applicability

of eDNA to detect New Zealand fish species, we used the eDNA technique alongside more traditional assessment methods (electrofishing, spotlighting) to determine the presence, or indicate the absence, of brown trout following a rotenone (piscicide) application. The use of eDNA may address some of the requirements of the spotlighting and electrofishing such as labour required, the need for specialised equipment and training, and differences between the methods in detectability depending on habitat. Specifically, we tested the hypothesis that eDNA will effectively detect the presence of brown trout in streams of the Zealandia wildlife sanctuary.

Methods

Study area and sample site selection

The Zealandia sanctuary is located in the lower North Island of New Zealand, near the city of Wellington. The sanctuary is a 252 ha retired municipal water supply catchment with a series of small streams draining into an upper and lower reservoir formed by two dams (Fig. 1).

Rotenone application

Areas for rotenone application were selected based on trout presence/absence inferred from repeated electric fishing (Jowett and Richardson, 1996) and/or spotlighting (McCullough and Hicks, 2002) of each reach, the presence of putative trout barriers, and stream flows high enough to support trout. The discharge of the largest tributary (TLH) was never above 7 L/s when measured and the flow rates of the untreated streams were much lower. Many of the smaller first and even some of the second order streams are ephemeral and only flow during winter thus were

unlikely to contain trout. Where possible, banded kokopu, *Galaxias fasciatus*, the only native fish known to live in the parts of the streams to be treated and the taonga (culturally valued) species koura (freshwater crayfish), *Paranephrops planifrons*, were removed from the treatment areas, held in cages above the treatment area and then returned once the rotenone had dissipated or become inactive.

The majority of the flow of the upper reaches (and middle reaches for the larger stream) of the true left (i.e., left tributary if facing downstream) and true right treatment streams were treated with rotenone on 22 February 2011 using a divert, dose (cube-root-slurry containing 6–9 % rotenone in water) and discharge (200 µg/L rotenone) system using bunds and mixing tanks. The smaller stream was dosed for 4 hours and the larger stream for 7 hours. Small inflows were also sprayed at the same time with three times the fully mixed concentration of rotenone by four people using backpack sprayers to produce a final instream concentration of approximately 200 µg/L rotenone. The upper reservoir was treated at the same time using shore-based and boat-mounted rotenone spraying equipment. Slow downstream travel times of rotenone meant that the lower section of the larger stream may not have been completely treated on February 22 despite limited use of a booster station half way down the stream later in the afternoon. It was therefore decided to re-treat the lower section of the larger stream on February 23 using the booster station.

Pre-rotenone numbers of trout upstream of each water sample were estimated by dividing the two main tributaries into a series of 200 m reaches by the placement of nets immediately before the application of rotenone. The nets were

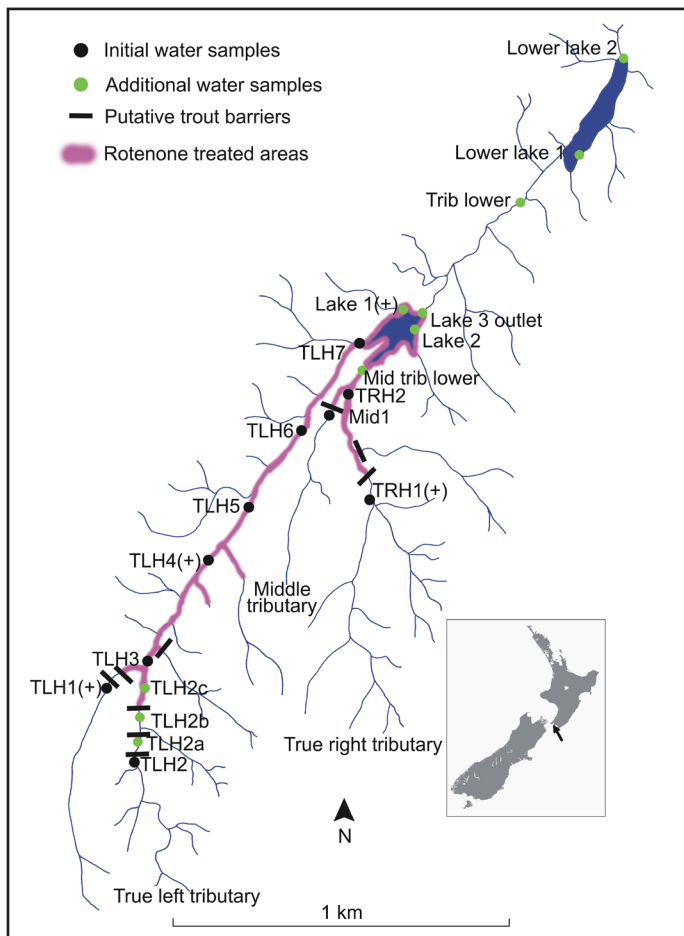


Figure 1. Water sample sites and position of putative trout barriers in Zealandia Sanctuary. (+) after the sample site name designates samples which were positive for brown trout DNA in the second round of post rotenone samples (August 5, 2011). Inset map shows location of Zealandia sanctuary in New Zealand.

removed three days after the rotenone application. Each reach was monitored by observers and dead fish were collected from within reaches and from block nets each day until nets were removed. The number of dead fish collected from each reach and the distances from the source of the tributaries was used to calculate the number of fish above each sampling site (Table 1).

Field Sampling for eDNA

Sampling sites were selected on the basis of trout having been caught after electrofishing or observed in spotlight

surveys. Sites were also selected in sections of the streams from which trout were inferred to be absent based on the presence of trout barriers such as waterfalls that prevented trout moving upstream into upper reaches of the waterways, and the absence of fish after repeated electrofishing and spotlighting (Fig. 2). Two litre water samples were collected from ten sites in the streams of Zealandia sanctuary for the pre-rotenone and two post-rotenone rounds of sampling (Fig. 1) in plastic containers that had not been previously used for the collection of any samples, and had been

Table 1. Sequences obtained from positive (band seen on agarose gel) and negative (no band seen) polymerase chain reactions from samples collected 21 February 2011, one day before the rotenone application (+ = band or sequence obtained, - no band or sequence obtained, +/- designates a relatively short sequence of very poor quality was obtained, see methods for a description of the trout abundance calculation, NC = no sample collected in the 21 February 2011 sampling round).

Sample	Band	Sequence or band obtained	Sequence BLAST match	Hypothesised trout presence (pre-rotenone)	Estimated number of brown trout upstream of sample site.
TLH1	-	+/-	Brown trout 86 %	Most unlikely	0
TLH2	-	-		Definitely absent	0
TLH2a	NC	NC		Unlikely	0
TLH2b	NC	NC		Unlikely	0
TLH2c	NC	NC		Most unlikely	0
TLH3	Faint	+/-	Brown trout 100 %	Rare trout	0
TLH4	+	+	Brown trout 100 %	Occasional trout	59
TLH5	+	+	Brown trout 100 %	Trout common	116
TLH6	+	+	Brown trout 100 %	Trout abundant	200
TLH7	+	+	Brown trout 100 %	Trout abundant	295
TRH1	-	-		No trout	0
TRH2	+	+	Brown trout 100 %	Occasional trout	47
Mid1	-	-		No trout	0
Mid trib lower	NC	NC		No trout	None collected
Lake 1	NC	NC		Occasional trout	30 + tributaries
Lake 2	NC	NC		Occasional trout	30 + tributaries
Lake 3 outlet	NC	NC		Occasional trout	30 + tributaries
Trib lower	NC	NC		Occasional trout	N/A
Lower lake 1	NC	NC		Occasional trout	N/A
Lower lake 2	NC	NC		Occasional trout	N/A

treated for 20 minutes in a 10 % bleach solution (final concentration of sodium hypochlorite = 0.4 % w/v) then dried before use. Water samples were collected on 21 February 2011, 18 May 2011, and 5 August 2011, one day before, and 85 and 164 days after, rotenone was applied to sections of the streams below the trout barriers to eradicate brown trout. As a live trout was found above the treatment area in May, three additional sites near where the trout was found (TLH2a, TLH2b and TLH2c) were added to the protocol for the August 5 samples. Further water samples were also taken on August 5 from reservoir and downstream sites to assess

the feasibility of obtaining eDNA from larger water bodies. Water samples were frozen at -20 °C, packed into polystyrene containers with dry ice and shipped to the Pacific Barcoding Research Laboratory (PBRL), at the University of Waikato.

Laboratory Analyses

All water samples were stored at -20 °C until needed for analysis. Samples were thawed by placing the 2 L containers in a 55 °C water bath and then filtered. Filtration equipment was sterilised by immersion in a 10 % bleach solution (final concentration of sodium hypochlorite = 0.4 % w/v) for 20 minutes followed by



Figure 2. True right barrier approximately 1 m high, upstream of which brown trout were assumed to be absent as none were caught despite repeated surveys over multiple years. Pool below the barrier was used as a rotenone application point.

two washes in distilled, deionised water and consisted of an inverted 3 L glass volumetric flask, with the base removed, placed on a 90 mm diameter perspex filter platform (Technical Glass Products, Dunedin). The volumetric flask and filter platform were held on a 90 mm diameter glass funnel using 25 mm tool clips. The funnel ran through a stopper which sat on a 2 L Büchner flask. We cut a 150 mm diameter GF/A glass fibre filter (pore size 1.6 μm , Whatman, Maidstone, Catalogue number 1820 150) to fit the filter platform. The 90 mm diameter filter was larger than the 55 mm diameter filter used by Jerde et al. (2011) as we found that the Zealandia water samples often clogged smaller diameter filters or took too long to process. We connected a vacuum pump (Cole Parmer, Vernon Hills, Model number EW 79200-05) to produce approximately 40 mmHg of negative pressure to the Büchner funnel to increase the filtration rate. Filters containing the filtrate were individually placed in 50 mL conical tubes (Corning,

Sigma-Aldrich, St. Louis) and frozen at $-20\text{ }^{\circ}\text{C}$ until the DNA could be extracted from the filters. We filtered 2 L of distilled, deionised water as negative controls.

Filters were thawed in batches of eight and the DNA extracted using Power Water kits (MoBio Laboratories, Carlsbad). The manufacturer's directions for extracting DNA were followed except that we halved each filter with scissors that had been dipped in 100 % ethanol and flamed to remove any residual DNA. We also increased the bead beating step from the manufacturer's recommended 5–10 minutes to break up the larger filters and we centrifuged the broken up filters for 6 minutes rather than the manufacturer's recommended 1 minute to release the extraction buffer from the homogenised filters. The two aliquots of extraction buffer were processed separately until the DNA was loaded on to the spin column at which point the two aliquots of solubilised DNA from each water sample were recombined and filtered

through a single column. DNA was eluted from the column using 100 μ L of the manufacturer's elution buffer.

The primers STF (AAG TTG GTG GGT AAA GAC GGA GC) and STR (TGA ATT TAA CCC CTC ATA CAT CAG C) were designed for this study using Primer3 (Rozen and Skaletsky, 2000) in Geneious (Drummond et al., 2010) to amplify a 148 nucleotide portion of the mitochondrial d-loop region from the brown trout using polymerase chain reactions (PCR). The primers did not amplify DNA extracted from banded kokopu muscle tissue, the only other fish species known to be present in the sanctuary streams.

Labware was treated by exposure to UV light for 10 minutes immediately before use and all reactions were set up in a laminar flow cabinet delivering HEPA filtered air. Reactions consisted of 10 μ L of PCR ready mix (iNtRON Biotechnology, Korea, catalogue number 25028), 0.5 μ L of 10 μ M/L primers STF and STR, 1 μ L of water and 8 μ L of DNA template. Reaction conditions were an initial denaturing step at 94 °C for 2 minutes, followed by 40 cycles of 94 °C for 30 seconds, 53 °C for 1 minute and 72 °C for 30 seconds, with a final extension step of 72 °C for 1 minute. A no template, negative control reaction and a positive control reaction (DNA extracted from brown trout tissue) were included in each reaction set, in addition to the no template extractions. Reaction products were run out on 2 % agarose gels in TBE buffer at 40 V for 2 hours and visualised using ethidium bromide (0.5 mg/L). Pre-rotenone PCRs and the entire second batch of post-rotenone PCRs (including reactions which did not show a band), were purified with ExoSAP-it (Affymetrix, Santa Clara, Catalogue number 78201) and sequenced on an ABI

3130XL DNA Sequencer. No reaction PCRs were sequenced for the first set of post-rotenone samples. Sequences were compared to those available on GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the Basic Local Alignment Search Tool (BLAST) algorithm.

Results

For the pre-treatment (21 February 2011), bands were seen in the agarose gels for all samples below the trout barriers and no bands were seen from PCRs conducted on samples taken above the putative trout barriers. We obtained high quality sequence data (e.g., Fig. 3) for a 100 nucleotide fragment (excluding the primer binding sites) for the trout d-loop region from five of the six water samples below the putative trout barriers. BLAST searches for the sequences matched with those for brown trout in GenBank (Table 1, Fig. 5). We obtained low quality sequence data from the weak band obtained from sample TLH3 (below the trout barriers). The sequence for TLH3 consisted of two fragments from each end of the d-loop region we sequenced (25 and 41 nucleotides long, Table 1). These two fragments were a 100 % match to brown trout sequences in GenBank (Fig. 5).

We also sequenced all band-negative PCRs from the pre-rotenone samples. No interpretable DNA sequence was obtained for any band-negative PCRs except that we obtained a fragment of very poor quality sequence from site TLH1 which is above the trout barriers (Table 1, Fig. 4). The corresponding BLAST search found the closest match to be brown trout (Fig. 5), albeit with a much lower sequence similarity (86 %) due to the poor quality of the sequence read.

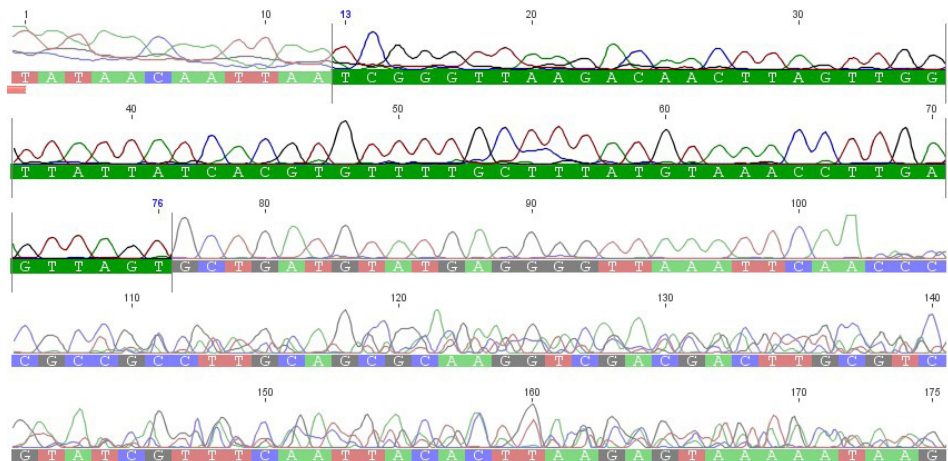


Figure 3. Electropherogram of high quality sequence from sample TLH7. The section between nucleotides 12 and 77 (highlighted) is a 100 % match to *Salmo trutta* in GenBank (accession number JN007645). Nucleotides 77 to 101 are the sequence for the reverse primer STR.

For the post-treatment (18 May 2011), no bands were observed on any of the agarose gels for this sampling period and PCRs were not sequenced. However, for the post-treatment (5 August 2011), an obvious band on an agarose gel was observed from site TRH1, although no other obvious bands were noted. For this time, all PCRs were sent for sequencing and we obtained brown trout sequences from three of the original ten sample sites (TRH1, TLH1 and TLH4), and

one brown trout sequence from one of the additional sample sites (Lake 1). Two samples (TLH2a and TLH7) both produced poor quality sequences that did not match any sequence in the GenBank database. Complete sequencing results for all sites are listed in Table 2.

Discussion

For the pre-rotenone treatment period, we were able to obtain high quality

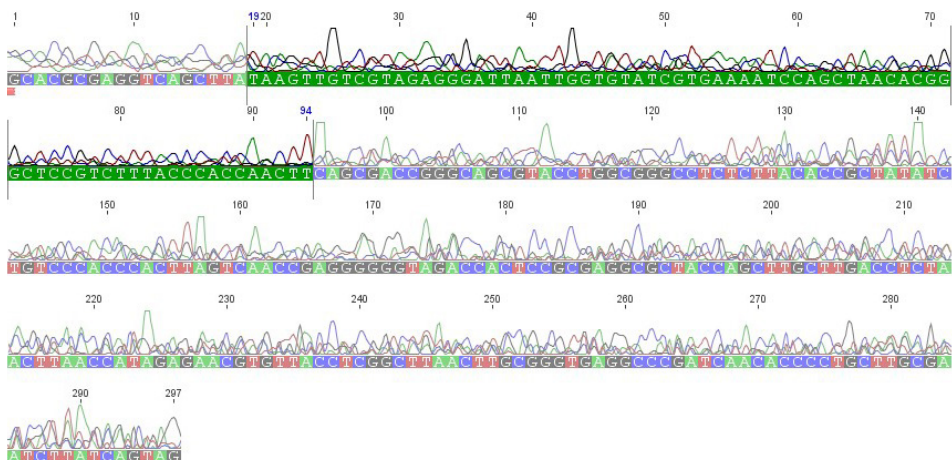


Figure 4. Electropherogram from sample TLH1. The section between nucleotides 18 and 95 (highlighted) is an 86 % match to *Salmo trutta* in GenBank (accession number JN007646) with six mismatches and five gaps. The multiple peaks at each nucleotide position are often indicative of low signal strength relative to noise.

Sample	Sequence								
Salmo trutta									
JN007645	TGAATTTAAC	CCCTCATACA	TCAGCACTAA	CTCAAGGTTT	ACATAAAGCA	AAACACGTGA	TAATAACCAA		
TRH2
TLH7
TLH5
TLH4
TLH3
TLH1	T.....C.....	..A.....
Sample	Sequence								
Salmo trutta									
JN007645	CTAAGTTGTC	TTAACCCGAT	TAATTGTTAT	ATCAATAAAA	CTCCAGCTAA	CACGGGCTCC	GTCTTTACCC	ACCAACTT	
TRH2A	
TLH7	
TLH6	
TLH5	
TLH4	
TLH3	
TLH1	M.....	G..GAGG...G.G.	...G-.G...	A.....	

Figure 5. Alignment of trout sequences from Zealandia water samples collected on 21 February 2011 (one day before the rotenone application), “-” designates no sequence obtained for that portion, “.” designates a nucleotide that is identical to the reference sequence JN007645, a portion of a 459 nucleotide control region sequence from a brown trout downloaded from Genbank.

sequences from five of the six samples collected from areas of Zealandia streams known to contain trout and a low quality sequence from sample TLH3 at the upper limits of the known trout distribution in the stream. The weak band in the gel and the poor quality sequence may reflect the low biomass of fish upstream of TLH3. We also obtained poor quality sequence from the band-negative PCR conducted on the sample collected from site TLH1, where brown trout were thought to be absent due to barriers such as waterfalls preventing upstream access. The closest match for the sequence obtained was brown trout (86 %), although the sequence signal strength (the heights of the peaks on the electropherograms) was low relative to the background “noise”. The absence of a band on our gel and the poor quality sequence indicates that there was possibly a low fish biomass upstream of TLH1. This positive sequence result suggests at least some trout may have been present above the trout barriers in the area of the stream that was not treated with rotenone. However we have now carried out electric fishing and spotlight surveys of the streams in February and October 2012 and no trout have been found. For the post-rotenone treatment period,

we did not observe any bands in the agarose gels and concluded that the application of rotenone had eradicated all trout from the streams. However, the capture of a live trout near site TLH2b following the first round of post-rotenone samples, and our positive eDNA results for sites TLH1, TLH2a, TLH4 and TLH7 in the left tributary and from site TRH1 in the right tributary in the second post-rotenone round of sampling suggest that at least one trout persisted in the tributaries upstream of the rotenone treated area. The positive sequencing results from some of the second post-rotenone sampling period, including those without obvious bands on agarose gels, suggest that sequencing may be desirable to confirm gel-negative results. Based on our field study, we were unable to assess the occurrence of any “false positives”; specifically, those instances where no live trout were actually present but DNA is detected (e.g. from DNA shed into the environment from carcasses). However, we assume persistence of DNA in the environment from carcasses to be minimal, as DNA has been shown to be detectable for only relatively short periods of time in the environment. For example, Dejean

Table 2. Sequences obtained from positive (band seen on agarose gel) and negative (no band seen) polymerase chain reactions from the second set of post-rotenone samples collected on 5 August 2011, 164 days after the rotenone application (+ = band or sequence obtained, - no band or sequence obtained, +/- designates poor quality sequence with low signal to noise ratio).

Sample	Band	Sequence obtained	Sequence BLAST match	Rotenone
TLH1	-	+	Brown trout 94 %	No
TLH2	-	-		No
TLH2a	+/-	-		No
TLH2b	-	-		No
TLH2c	-	+/-	Brown trout 97 %	Yes
TLH3	-	-		Yes
TLH4	-	+	Brown trout 95 %	Yes
TLH5	-	-		Yes
TLH6	-	-		Yes
TLH7	-	-		Yes
TRH1	+	+	Brown trout 97 %	No
TRH2	-	-		Yes
Mid1	-	-		Yes
Mid trib lower	-	-		Yes
Lake 1	-	+	Brown trout 96 %	Yes
Lake 2	-	-		Yes
Lake 3 outlet	-	+/-	Brown trout 100 %	Yes
Trib lower	-	-		No
Lower lake 1	-	+/-	Brown trout 97 %	No
Lower lake 2	-	-		No

et al. (2011) found that DNA was detectable for less than one month after tadpoles were removed from aquaria and sturgeon were removed from artificial ponds. Additionally there is the risk of the transfer of trout DNA by birds such as shags, *Phalacrocorax* spp. defecating in the water as prey DNA has been shown to amplifiable from faeces (Murray et al., 2011) or by humans transferring fish as the trout eradication was opposed by some members of the community (see for example, http://www.kiwiblog.co.nz/2010/10/dunne_v_zealandia.html). An added complication is that as the ratio of target DNA to other DNA decreases, false positives are more likely to be generated (Wilcox et al., 2013).

False negatives (failure to detect trout when they are present) are likewise a potential issue and the false negative rate is likely to be affected by a number of factors such as fish densities, sampling density, minimum detectable amounts of

DNA, and the flow rates of streams. For example Moyer et al. (2014) found that the false negative rate of African jewel fish detection was higher when fish densities were lower, water temperatures were higher and water samples were collected from the middle of the water column. Because of false negative reactions, Moyer et al. (2014) also found that it was necessary to increase the volume of water filtered to detect African jewel fish at low densities, requiring the filtration of hundreds of litres of water to detect fish at densities of < 0.32 fish/m³. Thus monitoring the success of fish eradication using eDNA will require a sound knowledge of false negative rates especially at low densities of fish.

Eradication of introduced species is facilitated by the early detection of incursions and accurate data on the extent of the species' range (Myers et al., 2000). Furthermore, detection of low densities of individuals would allow for the more

targeted application of control measures to specific regions (Myers et al., 2000). Silver carp were detected in waterways in the Chicago, Illinois area from their DNA shed into the environment several months before any carp had been physically observed (Jerde et al., 2011). Thus, detecting species from their DNA shed into the environment may be a more sensitive method of monitoring the distributions of freshwater fish species compared with methods such as electrofishing and spotlighting. Our detection of brown trout DNA above putative trout barriers in Zealandia's waterways suggest that the distribution of brown trout within the sanctuary may be more extensive than previously thought. In these cases eDNA would be a valuable tool to help eradication and restoration efforts to specific areas in the sanctuary. For example, the availability of eDNA data during the planning phase of the rotenone application in Zealandia could have aided in the selection of areas for treatment by providing additional evidence for the presence of trout.

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